

β -Adrenergic regulation of c-fos gene expression in an epithelial cell line

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Stimulation of β -adrenoreceptors in the RSMT-A5 epithelial cell line is accompanied by an early and transient increase in the expression of the proto-oncogene c-fos. Maximal induction was at 30 min, returning to basal levels after 2 h. Similar results were obtained when cells were incubated with 8-bromo-cAMP. The induction of c-fos is specific since the expression of p53, a transformation-related gene, is not modulated by isoproterenol or 8-bromo-cAMP. The increase in c-fos gene expression is not associated with proliferative activity in these epithelial cells.

β -Adrenoreceptor; Protooncogene c-fos; cyclic AMP; Epithelia; (RSMT-A5)

1. INTRODUCTION

Stimulation of β -adrenergic receptors by isoproterenol in vitro, in dispersed acinar cell aggregates of rat salivary glands, has been shown to influence a variety of intracellular processes such as protein production, phosphorylation, protein N-glycosylation and exocrine protein secretion [1–4]. In addition, in vivo chronic stimulation of rats with isoproterenol results in hyperplasia and hypertrophy of the parotid and submandibular glands [5]. Recently we have investigated the possible role of the proto-oncogene c-fos in such responses in rat parotid acinar cells after stimulation of β -adrenoreceptors [6,7]. c-Fos, the cellular homolog of the FBJ murine osteosarcoma virus, has been shown to be induced after stimulation of a variety of agents in many cells of mesenchymal derivation [8–11]. However, very little is known

about the expression of this proto-oncogene in epithelial cells.

In the present study we have made use of an epithelial cell line, RSMT-A5, developed from rat submandibular glands after treatment with methylcholanthrene [12]. RSMT-A5 cells possess functional β -adrenergic receptors since stimulation by isoproterenol increased the cAMP content of the cells by 130-fold [13]. In previous reports, using short-term preparations of enzymatically dispersed rat parotid acinar cells, we have shown that c-fos gene expression was induced by isoproterenol but this induction did not correlate with DNA synthesis [6,7]. Since acinar cell lines are not currently available, we have used the RSMT-A5 salivary epithelial cell line to gain more insight into the possible role of c-fos induction after stimulation of β -adrenergic receptors and its relevance to epithelial cell proliferation.

2. MATERIALS AND METHODS

2.1. Cell cultures

RSMT-A5 were derived from rat submandibular gland as described [12,13] and were grown in McCoy's 5A medium, sup-

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plemented with 10% fetal bovine serum (FBS, Hyclone) and 100 $\mu\text{g}/\text{ml}$ each of penicillin G and streptomycin sulfate, at 37°C in a CO₂ incubator. For experiments presented here, RSMT-A5 cells were used between passages 27 and 45, seeded onto 100-mm Petri dishes (Falcon) at a density of $0.5 \times 10^5/\text{cm}^2$, and allowed to grow to near confluence. Thereafter, the cells were incubated in the presence or absence of the β -adrenoreceptor agonist isoproterenol (10 μM , Sigma) or 8-bromo-cAMP (1 mM, Sigma) in the same medium supplemented only with 2% FBS for various time intervals. For β -adrenergic blocking experiments, cells were preincubated for 15 min in the presence of 50 μM propranolol (Sigma) before addition of the agonist. At the appropriate time intervals, the cells were frozen in liquid nitrogen and stored at -70°C, until used for RNA isolation. The incorporation of [methyl-³H]thymidine into DNA was studied after addition of the labelled nucleotide for 30 min following each indicated time point. Aliquots of

cells were made 10% (w/v) in CCl₃COOH and radiolabel in the precipitate was detected by liquid-scintillation spectrometry. Protein was determined by the Bio-Rad protein assay. HSG-PA were derived from human submandibular glands as described [14] and were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% newborn calf serum and 100 $\mu\text{g}/\text{ml}$ each of penicillin G and streptomycin sulfate, at 37°C in a CO₂ incubator. Cells were used between passages 54 and 60. The same experimental conditions used for RSMT-A5 cells were also applied for HSG-PA cells.

2.2. RNA isolation and Northern blot analysis

Cells were collected by scraping dishes in 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.1 M β -mercaptoethanol, 0.5% sarkosyl, and disrupted by 8 strokes in a glass homogenizer. Total RNA was isolated by the guanidinium/cesium chloride method [15]. Equal amounts of

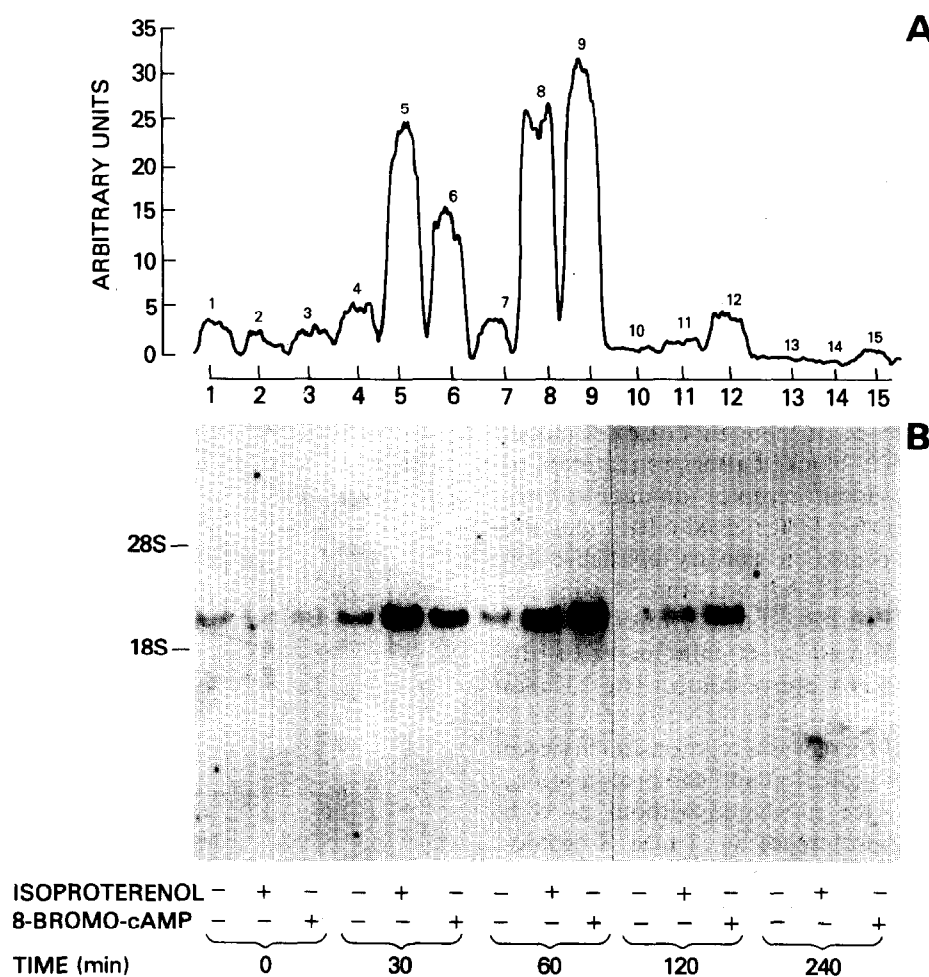


Fig.1. Effect of isoproterenol and 8-bromo-cAMP on c-fos expression in RSMT-A5 cell line. Isoproterenol (10 μM) or 1 mM 8-bromo-cAMP were added to RSMT-A5 cells growing in 2% FBS for the various time points indicated in the figure. 20 μg RNA were applied per lane and analyzed by Northern blot hybridization. The autoradiograms of the blots were exposed for 4 days. The autoradiogram (B) was subjected to densitometric analysis (A).

RNA from each sample were denatured for 15 min at 70°C and electrophoresed in 2.2 M formaldehyde/1.2% agarose gels [16]. After visualizing the RNA by ethidium bromide staining, the RNA was blotted onto nitrocellulose filters in 10 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M Na citrate, pH 7.4) for 20 h [17]. The transferred RNA was hybridized at 42°C for 16–18 h to nick translated *c-fos* cDNA, and subjected to autoradiography. Thereafter, the labelled probe was removed in a buffer containing 0.01 M Tris, pH 7.5, for 20 min at 80°C and re-hybridized with p53 cDNA, as described [18,19]. Filters were then again subjected to autoradiography at –70°C. Densitometric scanning of resulting autoradiographs was accomplished using a GS300 scanning densitometer (Hoefer Scientific) attached to an Omniscribe D-5000 strip-chart recorder. Sizes (kb) of mRNA were estimated by the 28 S and 18 S ribosomal RNA markers.

3. RESULTS

RSMT-A5 cells were incubated either with 10 μ M isoproterenol or 1 mM 8-bromo-cAMP as described in section 2. Fig. 1A and B shows that the level of *c-fos* mRNA significantly increased (~5.5-fold) above control levels after 30 min exposure of cells to either agent (lanes 4–6) and remained at that level after 60 min (lanes 7–9). Following this early induction, the steady-state levels of *c-fos* mRNA gradually declined to basal levels (lanes 10–15). *c-Fos* transcripts were not detectable in cells incubated with either isoproterenol or 8-bromo-cAMP for 4–24 h (not shown). The effect of isoproterenol on *c-fos* induction was completely blocked after pretreatment of cells with the β -adrenoreceptor antagonist propranolol (not shown). That indeed *c-fos* induction by cyclic AMP occurs in salivary epithelial cell lines, other than RSMT-A5, was studied using the HSG-PA cell line. Addition of 8-bromo-cAMP in these cells for 30 and/or 60 min increased the levels of *c-fos* mRNA above control (fig. 2, lanes 1–3).

To determine if there was a relationship between the levels of *c-fos* induction and cell proliferation, RSMT-A5 cells were incubated without or with either 10 μ M isoproterenol or 1 mM 8-bromo-cAMP for various time points and the incorporation of [*methyl*-³H]thymidine into DNA followed (fig. 3). DNA synthesis was decreased after incubation of cells with either isoproterenol or 8-bromo-cAMP for 2 h. After 4 h, [*methyl*-³H]thymidine incorporation in isoproterenol-treated cells returned to control levels and remained so up to 24 h (not shown). At all time points tested (1–24 h) 8-bro-

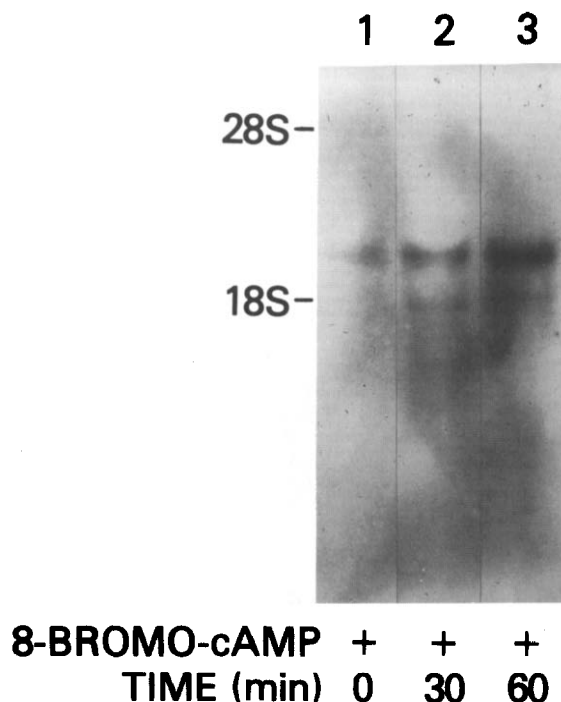


Fig. 2. Effect of 8-bromo-cAMP on *c-fos* expression in HSG-PA cell line. 1 mM 8-bromo-cAMP was added to HSG-PA cells growing in 2% newborn calf serum for the various time points indicated in the figure. 20 μ g of RNA were applied per lane and analyzed by Northern blot hybridization. The autoradiograms of the blots were exposed for 4 days.

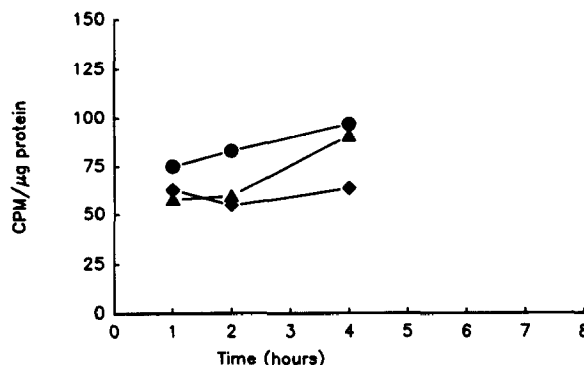


Fig. 3. Effect of isoproterenol or 8-bromo-cAMP on [*methyl*-³H]thymidine incorporation into DNA. RSMT-A5 cells were incubated in the presence or absence of 10 μ M isoproterenol or 1 mM 8-bromo-cAMP for the time periods indicated as described in section 2. The values (average of three experiments) are presented as [*methyl*-³H]thymidine incorporation per microgram protein. (●—●) Control; (▲—▲) isoproterenol; (◆—◆) 8-bromo-cAMP.

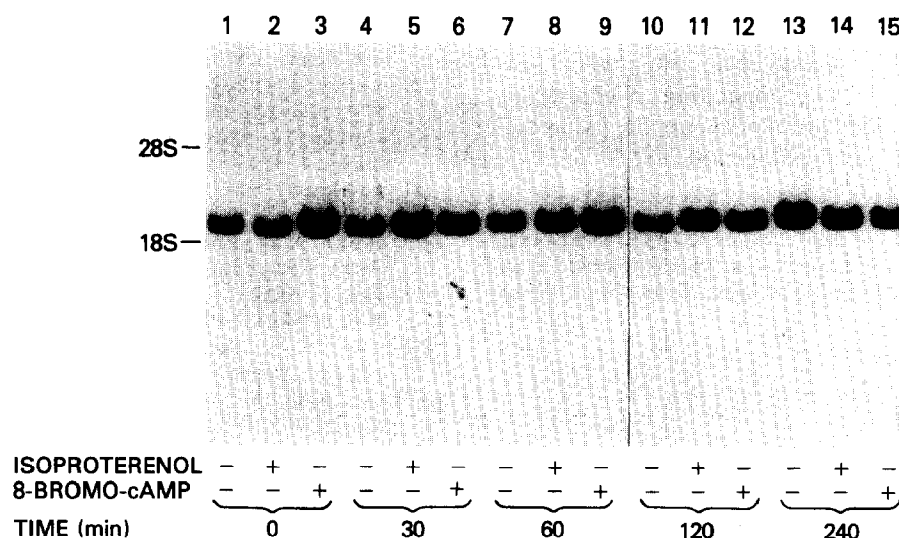


Fig.4. The effect of isoproterenol and 8-bromo-cAMP on p53 mRNA levels. The Northern blots shown in fig.1 were re-hybridized with ^{32}P -labelled p53 cDNA as described in section 2. The autoradiographic exposure time was 4 days. Positions of ribosomal RNA markers are indicated.

mo-cAMP-treated cells displayed reduced levels of [*methyl*- ^3H]thymidine incorporation compared to control cells.

To evaluate the specificity of the induction of c-fos expression, we also examined the expression of p53, a transformation-related gene [20]. p53 was highly expressed in RSMT-A5 cells, there were no detectable changes in its expression after exposure to either isoproterenol or 8-bromo-cAMP (fig.4).

4. DISCUSSION

The results presented in this study clearly indicate that after stimulation of β -adrenergic receptors in RSMT-A5 cells c-fos mRNA steady-state levels increased transiently. This transient c-fos induction appears to be mediated by cAMP since β -adrenergic receptor activation elevates cAMP levels in these cells [13] and the cAMP analog 8-bromo-cAMP exerts a comparable effect on c-fos gene expression. The effect of isoproterenol and 8-bromo-cAMP on c-fos appears to be relatively specific because expression of p53, a putative oncogene which, like c-fos, also encodes for a nuclear protein [19], was unchanged by β -adrenoreceptor stimulation. This finding is unlike the observation made with dispersed parotid acinar cells, where treatment of the cells for 60 min with

10 μM isoproterenol induced both p53 and c-fos gene expression [6]. In addition c-fos gene expression was increased in HSG-PA cells after incubations with 8-bromo-cAMP. These cells have been shown to differentiate into myoepithelial cells after they were cultured in the presence of dB-cAMP [14].

In the RSMT-A5 cells, as in dispersed rat parotid acinar cells [6,7], the induction of c-fos gene expression does not seem to correlate with the induction of proliferative activity. Similarly, it has been shown that in A431 cell line c-fos induction is not strictly correlated with proliferation. Rather, it is a primary consequence of growth factor receptor interactions [21]. It has also been observed in NIH 3T3 cells that c-fos is inducible throughout the cell cycle [22]. Furthermore, in normal human peripheral blood lymphocytes, c-fos induction can occur without subsequent proliferation [23]. Our data support these earlier observations and extend the conclusions to a glandular epithelial cell line.

Thus, it would appear that, in many diverse cell types c-fos expression is more related to the differentiated functional state of the cell rather than to its growth characteristics.

The induction of c-fos in RSMT-A5 by β -adrenergic receptor stimulation through cAMP-mediated mechanisms is intriguing. This glandular

epithelial cell line (RSMT-A5) provides an excellent system for investigating the possible involvement of c-fos in mediating β -adrenergic receptor-cyclic AMP coupled events on tissue differentiation and cell proliferation.

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